



Faculty of Resource Science and Technology

**IDENTIFICATION OF ENTEROBACTERIACEAE FROM WATER SAMPLES
COLLECTED FROM LONG BEDIAN, SARAWAK**

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Identification of Enterobacteriaceae in water sample collected from

Long Bedian, Sarawak

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Final Year Project 2 (STF 3015) course

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DECLARATION

I hereby declare that this study entitled “Identification of Enterobacteriaceae in water sample collected from Long Bedian, Sarawak” is based on my original work and it has not been or concurrently submitted for any other degree at UNIMAS or other higher learning institutions.

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LIST OF ABBREVIATIONS

E. coli – *Escherichia coli*

°C – Degree Celcius

UTI – Urinary tract infection

UPEC – Uropathogenic *E. coli*

ml – milliliters

µl – microliters

NA – Nutrient Agar

EMBA – Eosin Methylene Blue Agar

TSI – Triple Sugar Iron

KIA - Kligler Iron Agar

API – Analytical Profile Index

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Identification of Enterobacteriaceae from water samples collected from Long Bedian, Sarawak

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ABSTRACT

Enterobacteriaceae is a large family of gram-negative bacteria. The more familiar bacteria in this family are *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella* and *Yersinia pestis*. This family also consist of disease-causing bacteria such as *Proteus*, *Serratia*, *Citrobacter* and *Enterobacter*. The infection caused by Enterobacteriaceae can be transmitted through the gastrointestinal tract, urinary tract and also respiratory tract. Long Bedian is a village situated at Baram. There are about 1,500 people living in this village. Besides, there are also tourists who go to Long Bedian for the natural environment all year long. The water from the river is their main source of water for daily living. Thus, the aim of this study is to isolate the bacteria found in the water sample collected from river in Long Bedian. The species of Enterobacteriaceae present in the water sample were identified to study the hazard pose by these Enterobacteriaceae to the public. From the result obtained, there were majority of *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Serratia*, which are opportunistic pathogen. Therefore, prevention step is suggested in order to secure the health of the people in Long Bedian, Sarawak.

Key words: Enterobacteriaceae, diseases, identification, public health

ABSTRAK

Enterobacteriaceae adalah dari kumpulan taksonomi besar bakteria gram-negatif. Bakteria yang agak terkenal dalam kumpulan taksonomi ini adalah *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella* dan *Yersinia pestis*. Kumpulan taksonomi ini juga mempunyai bakteria yang menyebabkan penyakit seperti *Proteus*, *Serratia*, *Citrobacter* dan *Enterobacter*. Penyakit yang disebabkan oleh Enterobacteriaceae boleh menjangkit melalui saluran pencernaan, saluran kencing dan saluran pernafasan juga. Long Bedian adalah satu kampung yang berada di Baram. Ia mempunyai lebih kurang 1,500 orang yang tinggal di kampung tersebut. Selain itu, ia mempunyai pelancong yang melawat ke Long Bedian untuk alam semula jadi sepanjang tahun. Sumber air dari sungai adalah sumber air utama untuk hidup seharian mereka. Oleh itu, tujuan kajian ini adalah untuk mengasingkan bakteria yang terdapat dalam sampel air yang dikumpul dari sungai di Long Bedian. Spesies Enterobacteriaceae yang terdapat di dalam sampel air telah dikenal pasti untuk mengkaji bahaya Enterobacteriaceae ini kepada orang awam. Daripada keputusan yang diperolehi, majoritinya adalah *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Serratia* yang merupakan patogen oportunistik. Oleh itu, langkah pencegahan telah dicadangkan untuk menjamin kesihatan penduduk di Long Bedian, Sarawak.

Kata kunci: Enterobacteriaceae, penyakit, mengkaji, kesihatan awam

INTRODUCTION

Enterobacteriaceae are a large family of gram-negative bacteria. The more familiar bacteria that are in this family are *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella* and *Yersinia pestis* (Epstein, 2015). This family also includes other disease-causing bacteria such as *Proteus*, *Serratia*, *Citrobacter*, and *Enterobacter* (Guentzel, 1996). There are several characteristics of Enterobacteriaceae. The most commonly known characteristic is they are a short rods shape, which is typically about 1-5 μm in length (Young, 2006). Enterobacteriaceae are non-sporulating bacteria. Besides that, Enterobacteriaceae only have simple nutritional requirements, researcher uses MacConkey agar in order to isolate and differentiate organisms of this family (Selective and Differential Media, n.d.). Enterobacteriaceae are catalase positive and oxidase negative (Archarya, 2013). Archarya (2013) also stated that Enterobacteriaceae usually can reduce Nitrate to Nitrite. According to Mayer and Makela (1976), these bacteria contain a characteristic antigen which is known as the enterobacterial common antigen.

Escherichia coli is the most commonly known and well-studied Enterobacteriaceae. It is commonly found in the intestinal tracts of humans and animals (Morris, 1992). According to Li et al. (2012), most of the *E. coli* is not infectious but some serotypes are pathogenic and can cause serious food poisoning in humans. The harmless strains are part of the normal flora of the gut, they benefits their host by providing vitamin K, and also prevent the pathogenic bacteria from establishing within the intestine (Li et al, 2012). Besides, majority of pathogenic strains *E. coli* that cause disease are transmitted through fecal-oral transmission (Li et al., 2012). According to Edberg, Rice, Karlin and Allen (2000), for a limited amount of time *E. coli* are able to survive external of the body, which in turn making them an ideal indicator organism to test environmental samples for fecal contamination. The bacterium can also be grown easily and inexpensively in a laboratory

setting which makes it the most widely studied prokaryotic model organism and an important species in the fields of microbiology and biotechnology, where it served as the host organism for majority of work of recombinant DNA (Li et al., 2012).

The water samples collected for this research are from Long Bedian which is a village situated in Baram, Miri division. It occupies an area of about 15,000 sq m (Long Bedian beckons the adventurous, 2011). Currently, there are about 1,500 people living in this village. Majority of the population in Long Bedian are Kayans, with a mix of Kelabit, Kenyah, Morek, Penan and other smaller tribes (*Long Bedian – Sarawak*, n.d.). Besides the local community which consist of the elderly, very young kids and mothers, Long Bedian is a also recreation place open to the public all year long (Long Bedian beckons the adventurous, 2011).

The purpose of this research is to identify the species of Enterobacteriaceae that is in the water of Long Bedian. This is to pinpoint whether these Enterobacteriaceae will cause any disease to the community and also to the visitors to Long Bedian. This will help to protect the health of the community and visitor in Long Bedian. Besides, by finding out that the water in Long Bedian is safe, it can help to bring more visitors to Long Bedian and therefore improving the lifestyle of the community in Long Bedian.

Water sample is first collected from different source and area surrounding Long Bedian such as Tenyok River, Nyang Waterfall, and Nawan Waterfall which is near a recreation resort known as Tenyok Rimba Community Resort. After the water sample is collected, a series of procedure is carried out in order to isolate the bacteria in the water sample. These isolates can then be kept at -20°C in order to be used for other study again in the future. The isolates will then be used to grown to a larger amount and then a series of biochemical test

will be carried out in order to identify and study the Enterobacteriaceae in water samples collected from Long Bedian, Sarawak.

Objective of this study:

- a. To identify the species of Enterobacteriaceae found in the water samples collected from rivers in Long Bedian, Sarawak.
- b. To study the species of Enterobacteriaceae found in the water samples collected.

LITERATURE REVIEW

2.1 Introduction of Enterobacteriaceae

Enterobacteriaceae are a large family of gram-negative bacteria. They are small in size which is about 1-5 μm in length (Sherley, Gordon, & Collignon, 2003). Enterobacteriaceae is also a rod shaped bacteria (Chishti, Alam, & Kiessling, 2013). The family of bacteria is also well known for its oxidase negative properties whereby they will not undergo oxidation reaction with oxidase reagents (Hadar, Harman, Taylor, & Norton, 1983). According to Archarya (2013), Enterobacteriaceae are able to reduce nitrate to nitrite form. These bacteria are able to ferment glucose and other carbohydrates as well (Stiles & Ng, 1981). There are many genera that are present in the Enterobacteriaceae family, examples are *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Enterobacter*, *Yersinia*, and others (Guentzel, 1996). Enterobacteriaceae can be divided into opportunistic pathogens and true pathogens strains. Examples of true pathogens are *Salmonella*, *Shigella*, *Yersinia*, and some strain of *E. coli* (Small, Isberg, & Falkow, 1987).

E. coli is the most commonly known and well-studied Enterobacteriaceae. According to Morris (1992), *E. coli* is usually found in the intestinal tract of humans and animals. Basically there are about 0.1% of *E. coli* can be found in the gut. According to Li et al. (2012), harmless *E. coli* strains are part of the normal flora of the gut and it benefitted the host by producing vitamin K to it, besides it also prevent the pathogenic bacteria to establish within the intestine. Most of the *E. coli* species are not infectious but some serotypes are pathogenic and it can cause serious food poisoning to humans (Li et al., 2012). Li et al., (2012) also stated that majority of the pathogenic *E. coli* that cause diseases are transmitted through fecal-oral transmission route. According to Edberg, Rice, Karlin and Allen (2000), *E. coli* are able to survive outside of the body for a limited amount of time, which in turn making them an ideal indicator organism to test

environmental samples for fecal contamination. Besides that, Li et al. (2012) stated that the bacterium can be grown easily and inexpensively in a laboratory setting and this makes it the most widely studied prokaryotic model organism and an important species in the fields of microbiology and biotechnology, where it served as the host organism for majority of work of recombinant DNA. Another reason that *E. coli* is a widely studied organism is due to it has a fast doubling time of about 15-20 minute (Rosano & Ceccarelli, 2014).

2.2 Infection caused by *Escherichia coli*

Even though majority of *E. coli* are not infectious, but *E. coli* are also responsible for certain disease. The infection that can be caused by *E. coli* can be divided into three major types which are the gastrointestinal infections, urinary tract infections, and neonatal meningitis. In some cases, the virulent *E. coli* strains are also responsible for mastitis, septicemia, haemolytic-uremic syndrome, and gram-negative pneumonia (Md. Shahidul Islam, Khondkar, Md. Anwar UI Islam, 2010).

Gastrointestinal infections caused by *E. coli* are by a certain strains such as O157:H7, O104:H21, and O104:H4, which are responsible of producing potentially lethal toxins (Bommarius et al., 2013). Food poisoning caused by *E. coli* can result from eating unwashed vegetables or poorly butchered and undercooked meat. According to Petruzzello, Mawji, Khan and Marsden (2009), O157:H7 is also infamous for causing serious and even severe complications such as hemolytic-uremic syndrome. This particular strain of *E. coli* is also responsible for United States *E. coli* outbreak in 2006 due to fresh spinach (Rasko et al., 2008). Qin et al. (2011) stated that the O104:H4 strain is equally virulent and it is the strain behind the deadly *E. coli* outbreak in Europe on June 2011. The severity of illness varies considerably, it can be fatal to the young children, elderly or the immunocompromised, but the effect is often mild.

Majority of the urinary tract infections (UTI) with ordinary anatomy are caused by uropathogenic *E. coli* (UPEC). UTI is an ascending infection where the fecal bacteria colonize the urethra and spread up the urinary tract to the urinary bladder then to kidney, or the prostate in males (Sobel, 1997). Women are more likely to suffer ascending UTI due to their shorter urethra compared to men. UPEC uses P fimbriae to bind to the urothelial cells of urinary tract and then colonize the bladder.

2.3 History and Development of Long Bedian

During the war and the Japanese occupancy, deep in the tropical forest of Sarawak, a group of Ulu people planned a great migration (*Long Bedian – Sarawak*, n.d.). In the year 1946, members of that society moved out and came across a small canyon in Kuala Sungai Bedian or Sungai Durian. They then settled down and build a longhouse (Long Bedian beckons the adventurous, 2011). Their settlement was named after Long Bedian where the word “Long” means “Kuala”. The development of the Kayans’ lifestyle along with the outside world contributed to the preservation of “Long Bedian” till today (Long Bedian beckons the adventurous, 2011). After few years of living in Long Bedian, they soon abandoned their old religion and embraced Christianity (Long Bedian beckons the adventurous, 2011). This change had a great effect on the people of Long Bedian. Many socio-economic changes also occurred in the village. At the end of 1960, the community of Long Bedian was introduce to farming, planting coffee as well as to government policies such as attending formal education in national school in Long Bedian was made compulsory (Long Bedian beckons the adventurous, 2011).

Now, there are about 1,500 people living in this village. Majority of the population in Long Bedian are Kayans, with a mix of Kelabit, Kenyah, Morek, Penan and other smaller tribes (*Long Bedian – Sarawak*, n.d.). Majority of the local community consist of the elderly, very young kids and mothers, because majority of the men have left their hometown to make a living in the town area such as Miri. Long Bedian is also a recreation place open to the public all year long (Long Bedian beckons the adventurous, 2011).

2.4 Eosin Methylene Blue Agar

Eosin Methylene Blue was developed by Holt-Harris and Teague in 1916 (Lai & Cheeptham, 2013). EMBA is also known as Levine's formulation is a selective stain for gram-negative bacteria. EMBA is the mixture of two stains which is the eosin and methylene blue in the ratio of 6:1 (Archarya, 2013). EMBA is commonly used as a differential microbiological medium whereby it'll slightly inhibits the growth of gram-positive bacteria (Eosin Methylene Blue Agar, Levine, 2011). EMBA can also provide colour indicator to distinguish between organisms that ferment lactose such as *E. coli* and also those that do not ferment lactose such as *Salmonella* and *Shigella* (Lai & Cheeptham, 2013).

According to Lai and Cheeptham (2013), besides the dyes eosin Y and methylene blue, EMBA also contain peptone, lactose and sucrose. The dye methylene blue plays an important role in inhibits the growth of gram-positive bacteria (Lai & Cheeptham, 2013). While eosin is the dye which will responds to changes in pH, its colour changes from colourless to black under acidic condition (Archarya, 2013). The lactose and sucrose in the EMBA act as the energy source. Besides, these sugars are fermentable substrates which in turn will encourage the growth of some gram-negative bacteria (Lai & Cheeptham, 2013). The presence of sugars lactose and sucrose in EMBA and the ability of some bacteria to ferment lactose in the medium make the differentiation of enteric bacteria possible (Eosin Methylene Blue Agar, Levine, 2011). Lactose-fermenting gram-negative bacteria will acidify the medium and under acidic conditions the dyes produce a dark purple complex which is usually associated with green metallic sheen. According to Lai and Cheeptham (2013), the green metallic sheen indicates the vigorous fermentation activity in the medium. A slower rate of fermentation will result in lower amount of acid production will gives a brown-pink coloration of growth (Lai & Cheeptham, 2013).

2.5 API 20E system

API 20E test kit is an instrument that's use for the identification of enteric bacteria (Lindquist, 2010). This identification system provides an easy way to inoculate and read tests related to the members of the Family Enterobacteriaceae. The test strip of the API 20E system contains twenty mini wells which are used to test for twenty different biochemical tests (Holmes, Willcox, & Lapage, 1978). The tests that are included in the API 20E system are beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urea hydrolysis, deaminase, indole production, acetoin production, gelatinase, fermentation/oxidation reaction (Grisez, Ceusters, & Ollevier, 1991). After the result is observed from the test strip, it is converted into a seven-digit code which is called the Analytical Profile Index (API), the code will then be fed into the manufacturer's database through internet and it'll be able to produce the identification of organism present (Lindquist, 2010).

MATERIALS AND METHODS

3.1 Materials and apparatus

Nutrient Broth, Nutrient Agar (NA), Eosin Methylene Blue Agar (EMBA), Triple Sugar Iron (TSI) agar, Kligler Iron Agar (KIA), API 20E kit, petri dish, centrifuge tube, inoculation loop, inoculating needle, 70% ethanol, weighing machine, bunsen burner.

3.2 Source of water samples

The water samples were collected from Tenyok River, Nyang Waterfall and Nawan Waterfall which is near to Tenyok Rimba Community Resort.

3.3 Preparation of nutrient broth

390 ml of nutrient broth was prepared in order to regrow the isolated bacteria. The weight of nutrient broth powder needed to produce 390 ml of nutrient broth was calculated. The powder was then weighed with a weighing machine and obtained the exact weight of nutrient broth powder. Nutrient broth powder was then placed into a flask. Distilled water was measured with by using a measuring cylinder for 390ml and added into the flask. Nutrient broth powder was then mixed by shaking the flask and the flask was autoclaved at 121 °C for 2 hours.

3.4 Reviving isolates of presumptive Enterobacteriaceae with nutrient broth

Nutrient broth was left to cool to room temperature after autoclave is done. Then nutrient broth was poured into 39 different test tubes with 10 ml per tube. After the isolated bacteria sample melted, 39 different isolated samples was transferred into test tube with 100 µl each tube. The nutrient broths were then placed onto a shaker to overnight for the bacteria to regrow.

3.5 Preparation of nutrient agar

The weight of nutrient agar was calculated based on the volume of nutrient agar. Then the weight of nutrient agar powder was weighed on a weighing machine and obtained the exact weight. The powder was then transferred into empty flask and 250 ml of distilled water was added into the flask and mixed completely. The nutrient agar was autoclaved at 121 °C for 2 hours. After 2 hours, before the nutrient agar cool down, it was then poured onto petri dish and left to cool down to room temperature.

3.6 Grow bacteria onto nutrient agar

Petri dish that contain nutrient agar was separated into 2 sides by drawing a line on the petri dish. Each side of the petri dish was labelled according to the isolated bacteria sample. Inoculation loop is heated to red in colors, then cool down to room temperature and finally submerged into isolated bacteria sample. Isolated bacteria sample was streaked onto nutrient agar according to their label. Petri dish is then placed into a 37°C incubator overnight.

3.7 Preparation of Eosin Methylene Blue Agar (EMBA)

The weight of EMBA was calculated based on the volume of EMBA produced. EMBA powder was weigh with a weighing machine to obtain the exact weight of EMBA agar. Weighed EMBA powder was transferred into a flask and 500 ml of distilled water was added into it and mixed well. The solution was autoclaved at 121 °C for 2 hours. After autoclave and before the solution cool down to room temperature, the solution was poured onto petri dish in a laminar flow hood. Finally it was left to cool to room temperature.

3.8 Grow bacteria onto EMBA

Petri dish containing EMBA was labelled according to the isolated bacteria sample. Isolated bacteria sample was taken from the nutrient agar and streaked onto the EMBA using an inoculation loop. The petri dishes are then placed into 37°C incubator overnight for the bacteria to grow. This step was done repeatedly to obtain pure bacteria sample.

3.9 Preparation of Triple Sugar Iron (TSI) slant

The weight required to produce 200 ml of TSI agar was calculated. The TSI agar powder was then weighed using a weighing machine to obtain the exact weight. The powder was then transferred into an empty flask and 200 ml of distilled water was added into the flask and mixed completely. The flask is then placed into a microwave and heated until it was boiled. After the solution was boiled, it is poured into different test tubes with 10 ml each tube. It is then autoclaved at 121 °C for 2 hours. After autoclaved, the test tubes were placed in a slanting position and left to cool to room temperature.

3.10 Preparation of Kligler Iron Agar (KIA) slant

The weight required to produce 200 ml of KIA was calculated. The KIA powder was then weighed using a weighing machine. The powder was then transferred into an empty flask and 200 ml of distilled water was added into the flask and mixed completely. The flask is then placed into a microwave and heated until it was boiled. After the solution was boiled, it is poured into different test tubes with 10 ml each tube. It is then autoclaved at 121 °C for 2 hours. After autoclaved, the test tubes were placed in a slanting position and left to cool to room temperature.

3.11 Perform test with TSI slant and KIA slant

The procedure is the same for test using TSI slant and KIA. The inoculating needle was heated until its red and leave to cool to room temperature. Then the pure isolated bacteria sample was taken from EMBA using an inoculating needle. The inoculating needle containing isolated bacteria sample is then stabbed and streaked onto the TSI slant and KIA slant. After it is done, the TSI slant and KIA slant is placed into 37°C incubator overnight.

3.12 Perform oxidase test on each of the Enterobacteriaceae sample

Filter paper was cut into small pieces. The filter paper was then dropped with the oxidase reagent prepared. A sterile tooth pick was then used to remove a colony from the agar plate and then smeared on to the filter paper. The colour changes of the filter paper were observed within 10 seconds.

3.13 Preparation of Sodium Chloride (NaCl) solution

The weight required to produce 100 ml of weighed using weighing machine. The NaCl powder was then placed into an empty flask and 100 ml of distilled water was added into the flask. The powder was then mixed well with the distilled water using a mixer. The NaCl solution is then poured into 20 different test tubes with 5 ml each tube. The solution is then autoclaved at 121 °C for 2 hours. After autoclave is completed, the NaCl solution is left to cool to room temperature.

3.14 Perform bacteria identification with API 20E kit

A large colony was transferred from EMBA into the NaCl solution using inoculating loop. The mixture was then mixed completely using a vortex until there are without any clumps of floating bacteria. 5ml of distilled water was measured using a measuring cylinder and

poured into the tray and tilted it to evenly distribute the water. API 20E test strip were then placed into the tray.

The tray was hold in a flight angle up from the table top, and then the bacterial suspension was then inserted into each well of the API 20E test strip using a sterile pipette. The end of the pipette was touched to the side of the cupule allowing capillary action to draw the fluid into the well as the bulb is slowly squeezed. This is to eliminate any formation of bubbles in the wells. Each well was filled up to the neck. [CIT], [VP], and [GEL] were filled all the way up to the top of the well. LDC, ODC, ADH, H₂S, and URE were filled up until the neck with bacteria suspension, but then they were filled up to the top with sterile mineral oil. The tray was then covered with the cover provided. The API 20E test strip was then placed into 37°C incubator overnight.

After the test strip is incubated overnight. It was taken out and added a few proper reagents in to the specific compartments. 1 drop of James reagent was added into the IND well and the colour changes was read within a few minutes. 1 drop of VP1 and 1 drop of VP2 reagent was added into the [VP] well and the colour changes was read after 10 minutes. 1 drop of TDA reagent was added into the TDA well and colour changes was read within a few minutes.

The result of each well was then recorded into the paper provided in the API 20E kit. Those result were then key-in into a software provided by BioMerieux which is known as the APIweb. APIweb were then identified the potential organisms that present in each of the isolated bacteria sample.